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Expression and Evolution of the cation-coupled cotransporters in Aedes aegypti Larvae

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Abstract

Mosquitos are the most dangerous animals in the world, causing over 1 million human deaths annually by transmitting diseases such as Zika, malaria and dengue fever. A potential mosquito control strategy is to interfere with their unique osmoregulatory processes that include taking in salt and water with each bloodmeal and transitioning from aquatic larvae to terrestrial adults. We studied one group of osmoregulatory protein, known as cation-coupled cotransporters (CCCs). Using quantitative PCR, we measured the expression of *Aedes aegypti* CCCs in larvae exposed to varying levels of NH_4Cl, one transport protein (*ae*CCC3) was upregulated in 1 mM NH_4Cl. compound associated with waste products. In initial trials we found that In a separate project, we studied the family of membrane proteins that the CCCs belong to, the solute carrier 12 family (SLC12). By making phylogenetic trees, we assessed the evolution of the SLC12 family from a basal eukaryote. We also evaluated *Aedes* CCCs in the context of other animal CCCs. We found that *ae*CCC2 and *ae*CCC3 seem to result from an insect specific gene duplication event, and thus are not closely related to vertebrate transport proteins.

Introduction

Background

We have observed that knockdown of the $Na^+/K^+/2Cl^-$ cotransporter (NKCC, or aeCCC1) and aeCCC2 caused higher levels of hemolymph NH_4Cl in *Ae. aegytpi* larvae (1). As larvae live in aquatic environments that are often polluted with high concentrations of ammonia, it is probable that some protein is responsible for NH_4^+ movement in larvae. NH_4^+ can also substitute for K^+ in some cation-coupled cotransport, so these cotransporters serve as potential candidates for NH_4^+ movement.

At its most ancestral root, the NKCC seems to originate in animals. Other members of the SLC12 superfamily of proteins, such as CCC-interacting proteins (CIP1) and K^+Cl^- cotransporters (KCC), are seen in animals as well as other eukaryotes, including fungi and protists. Insects’ Na-dependent CCC duplicated after the divergence of vertebrates from inverteabrates, and a second duplication event is responsible for the third copy of CCC (2, Fig. 1). In mosquitos, aeCCC3 is seen significantly more in larvae than in adults (3,4), and this suggests that aeCCC2 and aeCCC3 may have a functional difference.

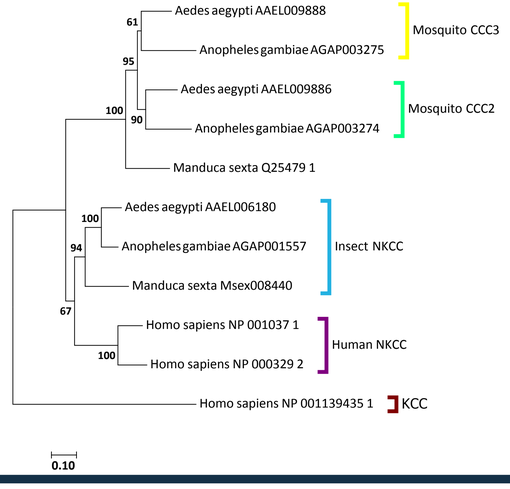

Fig. 1: Phylogenetic analysis of cation-chloride cotransporter (CCC) amino acid sequences by the unweighted pair group method with arithmetic mean. Gaps are excluded from the analysis. Modified from Piermarini *et al* 2017.

Methods

Animal Rearing: *Aedes aegypti* eggs were obtained from BEI Resources (LVP-IB12) or the lab’s colony. Eggs were hatched under a vacuum for 3 hours in ~3 cm standing 0.1% sea water and larvae were raised in 0.1% sea water for 72 hours. Larvae were fed daily with 3 parts TetraFin to every 1 part yeast extract *ad libitum*. The larvae were randomly sorted into 3 separate rearing conditions: 0 mM NH_4Cl, 1 mM NH_4Cl, and 5 mM NH_4Cl. The larvae were fed *ad libitum* once per day for another 48 hours. The water was changed once (48 hr) and the larvae were starved for the last 24 hours prior to RNA isolation. Colony was maintained with larvae raised in tap water, fed *ad libitum* daily as larvae and fed *ad libitum* with sucrose solution (10%) as adults. Pupae were transferred to an adult cage prior to eclosion. Blood feeding was performed with rabbit blood using a blood feeding apparatus. All organisms were stored in an incubator at 28 °C (75% RH) on a 12 hr light/day cycle.

RNA Isolation, cDNA synthesis, and qPCR: RNA was isolated from fourth instar larvae using Trizol reagent and protocol recommended by the manufacturer (ThermoFisher). RNA samples were purified with TURBO DNA-free (Ambion) and Clean & Concentrator (Zymo) according to manufacturer instructions. The concentrations of the RNA samples were measured by spectrophotometry. Total RNA (2.50 µg) was reverse transcribed to cDNA using the TaqMan polymerase kit (Applied Biosystems) under the following conditions: 50 µL/reaction, 25 °C for 10 min, 42 °C for 30 min, 95 °C for 5 min, 4 °C infinite hold. Template RNA without reverse transcriptase added to the reactions served as the negative control. Relative levels of transcript were quantified using SYBR Green mix (ABI), with primers for ribosomal protein S5 (aeRsp5) as the endogenous control and primers to detect aeNKCC1, aeCCC2, and aeCCC3. The template-free cDNA was used as the negative control. The qPCR ran as follows: 50 °C for 2 min, 95 °C for 10 min, 40x [95°C for 15 seconds, 60 °C for 1 min], with dissociation stage (95 °C for 15 seconds, 60 °C for 1 min, 95 °C for 15 seconds, 60 °C for 15 seconds) on the 7500 RT PCR System (ABI). DNA levels were quantified using dCt values and compared by ANOVA

Phylogenetic Analysis: Sequences with similarity to *Aedes aegypti* NKCC or CCC2 were obtained by BLAST search (NCBI) by queries with phyla of interest. Transmembrane domains were isolated using TOPCONS and sequence alignment in MAFFT. Preliminary trees were built in MAFFT by neighbor-joining on all gap-free sites and WAG substitution model (100 bootstrap trials).

Results

Did the duplication event responsible for insect CCC2/3 occur in crustaceans?

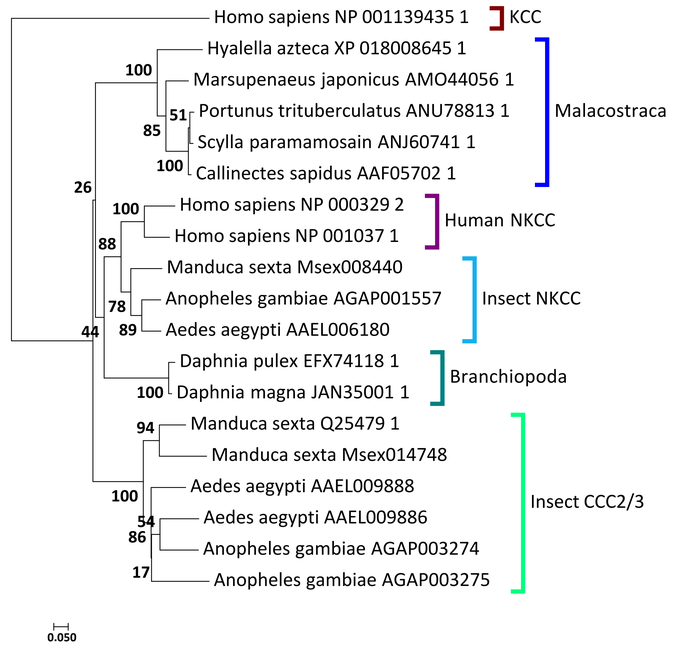

Fig. 2: Phylogenetic tree with crustacean and insect CCCs, built in MAFFT by NJ (100 bootstraps) on all gap-free sites. All crustaceans have only one copy of a gene with sequence similarity to *Ae. aegypti* NKCC. Branchiopoda group more closely to insect CCC and insect NKCC. Malacostraca group separate from the insect CCC2/3s and branchiopoda.

Are Na-dependent CCCs specific to animals?

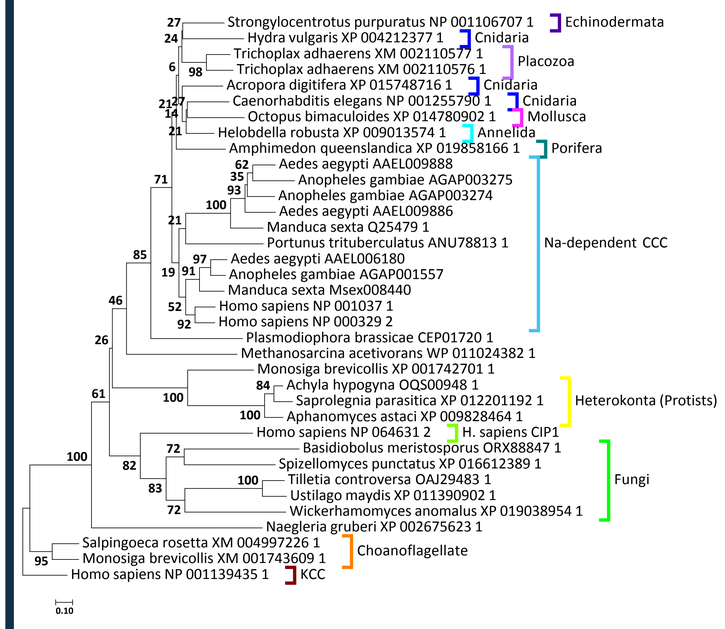

Fig. 3: Phylogenetic analysis of SLC12 family protein sequences across representatives of basal eukaryotes, fungi, and animal taxons. Built in MAFFT by NJ (100 bootstraps) on all gap-free sites. Fungi form a distinct clade of CIP1. Protists group separately from the fungi and animals, but as they do not group into the animal clades, then it seems that they do not possess Na-dependent cation cotransporters, and most likely possess protein that is CIP1-like. All animals seem to posses Na-dependent cation cotransporters, while protists and fungi do not.

Acknowledgments

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Results

Will NH_4Cl treatment upregulate any cation-coupled cotransporters in *Aedes aegypti* larvae?

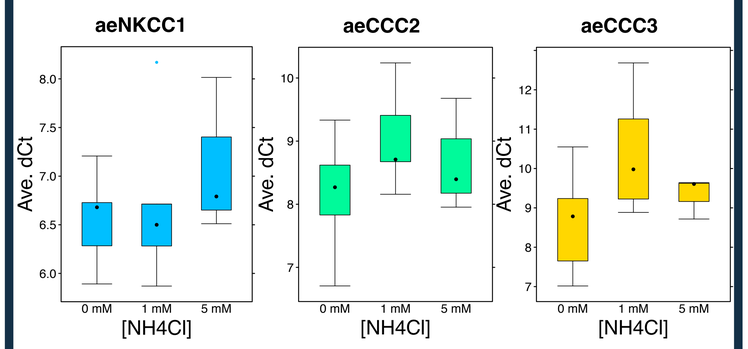

Fig. 4: Differential expression of three putative Na^+-coupled cation chloride cotransporter (CCC) gene transcripts across *Ae. aegypti* larvae exposed to varying levels of NH_4Cl for 72 hr. Smaller dCT values indicate higher expression. N = 9. Each sample was pooled from 8-10 larvae. Ribosomal RNA ‘Rps5’ = internal control. Error bars = SEM. For aeNKCC1: ANOVA, F = 0.7303, $df_{dev.}$ = 2, df_{error} = 12, p = 0.502. RQ_{1mM}= 1.1 , RQ_{5mM}=1.5 . For aeCCC2: ANOVA, F = 1.5756, $df_{dev.}$ = 2, df_{error} = 12, p = 0.2468, RQ_{1mM}= 1.8, RQ_{5mM}= 1.4. For aeCCC3: ANOVA, F = 2.871, $df_{dev.}$ = 2, df_{error} = 12, p = 0.096, RQ_{1mM}=3.5 , RQ_{5mM}=1.7 .

Summary

Phylogenetic data:

- Insects experienced a basal duplication event to the ancestral NKCC, and mosquitos and lepidoptera experienced a second duplication event later in their evolution.
- Vertebrates experienced a gene duplication to their NKCC independent of the arthropod duplication event.
- Crustacean NKCC did not experience a gene duplication event, and it may be that this ancestral NKCC gene has changed more to meet the needs of crustaceans as a result.
- Neither fungi nor protists have Na-dependent chloride cotransporters, and instead have CIP1 proteins, another member of the SLC12 family. These results agree with those published in (5).

qPCR data:

- No significant difference between these conditions, but aeCCC3 trends towards being expressed less in the 1mM and 5mM conditions than in the control condition.

Conclusion/Future Questions

Future Questions:

- Would a higher concentration of NH_4Cl induce changes in expression of aeCCC2/3?
- Will future replicates of this qPCR data reveal that the trend in aeCCC3 is significant?
- At what point in the evolution of animals was a true NKCC evolved?
- How do other insect species with multiple CCCs differ between their paralog sequences?

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